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Apoptosis in *Ichthyophthirius multifiliis* is associated with expression of the Fas receptor of theronts

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Abstract

The expression of type I membrane Fas receptors on the surface of *Ichthyophthirius multifiliis* (Ich) theronts and the possible association between Fas expression and theront apoptosis induced by the immune antibody was examined. Fas receptors were detected on the theront surface using fluorescein isothiocyanate-conjugated mouse monoclonal antibody against Fas. Fas-positive theronts significantly increased with time during *in vitro* incubation and with increasing theront concentration. Furthermore, the immune cutaneous antibody induced theront apoptosis; however, Fas ligand did not. A highly significant correlation was noted between theront Fas expression and immune cutaneous antibody-induced theront apoptosis. Numbers of apoptotic theronts increased with increasing number of Fas-positive theronts. The data indicated that theront apoptosis induced by immune cutaneous antibody appears to be positively correlated with the expression of Fas on the surface of Ich theronts.

Keywords: apoptosis, channel catfish, cutaneous antibody, Fas, FasL, *Ichthyophthirius*.

Introduction

The parasitic ciliate *Ichthyophthirius multifiliis* (Ich) infects most species of freshwater fish worldwide. Ich infection damages the gills and skin of fish and leads to high mortality. The parasite spreads rapidly from fish to fish, especially when the host is at high population densities (Paperna 1972; Nigrelli, Pokorny & Ruggieri 1976; Traxler, Richard & McDonald 1998). The life cycle of the parasite includes three stages: an infective theront, a parasitic trophont and a reproductive tomont (MacLennan 1935; Hines & Spira 1974).

Fish that survive an Ich infection develop an immune response and become resistant to reinfection (Hines & Spira 1974; Dickerson & Clark 1998). Antibodies from serum, mucus and skin culture fluid of fish immune to Ich cause immobilization of theronts and trophonts *in vitro* (Hines & Spira 1974; Clark, Dickerson, Gratzek & Findly 1987; Xu, Klesius & Shelby 2002). In a recent study (Xu, Klesius & Shoemaker 2005), apoptosis (programmed cell death) of theronts was noted when they were exposed to skin culture fluid from fish immune to Ich, which contained cutaneous antibodies against the parasite. Theront apoptosis was detected by fluorescent microscopy after staining with acridine orange and propidium iodide (PI) and with fluorescein isothiocyanate (FITC)-conjugated annexin using flow cytometry. The apoptotic theronts showed characteristic chromatin condensation, nuclear fragmentation and externalization of phosphatidylserine (PS) on the plasma membrane (Xu *et al.* 2005).

There is considerable interest in the cell surface receptor that induces apoptotic death when bound by its natural ligand or specific antibodies. Fas (also known as CD95 or Apo-1) is a type I membrane protein and expressed abundantly on the cell surface of various tissues (Nagata 1999). The ligand for Fas (FasL) is a type II membrane protein predominantly expressed by activated lymphocytes, monocytes and neutrophils. Binding of FasL to Fas or cross-linking of Fas with anti-Fas antibodies induces apoptosis of cells bearing Fas receptors (Itoh,

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Yonehara, Ishii, Yonehara, Mizushima, Sameshima, Hase, Seto & Nagata 1991; Nagata 1999).

Most studies of apoptosis have been conducted in higher eukaryotes (Zuñiga, Motran, Montes, Yagita & Gruppi 2002). However, there have been recent reports of apoptosis in simpler organisms such as the phytoplankton dinoflagellate *Peridinium* (Vardi, Berman-Frank, Rozenberg, Hadas, Kaplan & Levine 1999), the protozoans *Blastocystis* (Tan, Howe, Yap & Singh 2001), *Leishmania* (Das, Mukherjee & Shaha 2001; Lee, Berholet, Debrabant, Muller, Duncan & Nakhasi 2002), *Plasmodium* (Al-Olayan, Williams & Hurd 2002), *Tetrahymena* (Christensen, Sørensen, Beyer, Kristiansen, Rasmussen & Rasmussen 2001; Lu & Wolfe 2001) and *Trypanosoma* (Zuñiga *et al.* 2002). In the present study, we examined the expression of Fas receptors on the surface of Ich theronts and the possible association between Fas expression and theront apoptosis induced by immune cutaneous antibody.

Materials and methods

Fish and parasite

Channel catfish, *Ictalurus punctatus* (Rafinesque), reared at the United States Department of Agriculture, Aquatic Animal Health Research Laboratory, Auburn, Alabama, were used as host fish and maintained in tanks supplied with flowing dechlorinated water at 22–25 °C. Water quality was maintained by biological filtration, aeration and flow-through water supplied at 0.4 L min^{-1}. *Ichthyophthirius multifiliis* was isolated from an infected fish obtained from a local pet shop. The parasite isolate was maintained by serial transmission on channel catfish held in 50-L glass aquaria as described previously (Xu *et al.* 2002).

Theronts

Infective theronts were prepared for this experiment as described previously (Xu *et al.* 2002). Briefly, fish heavily infected with maturing trophonts were rinsed in tank water and the skin was gently scraped to dislodge the parasites. Isolated trophonts were placed in Petri dishes with dechlorinated water filtered through a 0.22-μm filter (Corning Costar, Corning, NY, USA) and allowed to attach. After replacing the water in the Petri dishes with fresh dechlorinated water to remove contaminating mucus, the trophonts were incubated for 18 h at 24 °C and theronts then harvested.

Four 15-mL centrifuge tubes were filled with theront solution at 10 mL per tube. After adding 5 mL phosphate-buffered saline (PBS; pH 7.2) to each tube, theronts were concentrated to 1 mL with a centrifuge (Beckman Coulter, Inc., Miami, FL, USA) at 60 ***g*** for 5 min, pooled into a 15-mL tube and washed by adding 10 mL filter-sterilized tank water. Numbers of theronts were counted in five 10-μL samples of the theront solution with the aid of a Sedgewick-Rafter cell (VWR Scientific Products, Atlanta, GA, USA).

Immunized fish and skin explants for culture

Five channel catfish, 18.2 ± 2.3 (mean ± SD) cm in length and 79.4 ± 4.7 g in weight, were exposed to 15 000 Ich theronts per fish in a 50-L tank for 1 h. The fish showed visible trophonts on the body surface 4 days after exposure to theronts. The infected fish were treated with formalin at a concentration of 100 mg L^{-1} (equivalent to 37 mg L^{-1} formaldehyde) for 1 h daily for 5 days to prevent Ich re-infection. The fish were then kept in the aquaria for 21 days to develop an immune response. Five fish from a tank not exposed to Ich theronts were used as non-immune controls. Both immunized and non-immunized fish were used to collect skin explants for culture as described previously (Xu *et al.* 2002). Briefly, the skin was collected from the lateral body wall of each fish and cut into 5 × 5 mm pieces. The excised tissues were washed three times with Hank's balanced salt solution and once with Medium 199 (Sigma Chemical Co., St Louis, MO, USA). Approximately 1 g of the excised skin and 2 mL culture medium were added to each well of a 6-well plate and allowed to incubate for 24 h. After culture, fluids from five fish were centrifuged at 228 ***g*** and 10 °C for 10 min, the supernatant was collected, pooled, heat inactivated at 56 °C in a water bath for 30 min and stored at −80 °C.

Determination of cutaneous anti-Ich antibodies in skin culture fluid

Cutaneous antibodies against Ich in skin culture fluid were determined with the theront immobilization assay (Xu *et al.* 2002) and an enzyme-linked immunosorbent assay (ELISA, Xu *et al.* 2005) described previously. Immobilization titre was the

highest dilution in which all theronts were immobilized. For ELISA, optical density (OD) readings in immune fluid two times greater than the OD readings in control fluid were considered as positive. The ELISA titre was the highest dilution in which an immune sample was positive.

Detection of apoptosis by fluorescein isothiocyanate conjugate of annexin V

The distribution of PS moves from the inner to the outer surface of the cell membrane during apoptosis (Vermes, Haanen & Reutelingsperger 1995). Theront apoptosis-associated movement of PS onto the external surface of the cell membrane was determined with an Annexin V Apoptosis Detection Kit (Immunotect; Beckman Coulter Corporation, Marseille Cedex, France). This kit contains FITC conjugate of annexin V that binds preferentially to PS with high affinity. Briefly, 100 μL binding buffer was added to 200 μL concentrated theront solution in a flow cytometer tube. Annexin V-FITC solution and PI were added to each tube following the manufacturer's instructions, mixed, and incubated in an ice bath for 15 min in the dark. The apoptotic cells in 2000 theronts were counted using the Coulter Epics flow cytometer (Beckman Coulter, Inc.) equipped with a 15 mW argon ion laser operating at 488 nm.

Detection of Fas on theronts

To detect Fas expression on the surface of theronts, 5 μL FITC-conjugated mouse monoclonal antibody against Fas receptor (FITC-Mab-Fas) (Immunotect) was added to 200 μL theront solution (approximately 4000 theronts) and incubated for 15 min. The amount of FITC-Mab-Fas (5 μL) and incubation time (15 min) selected for use in this study were based on the manufacturer's recommendations with modification after a series of tests (data not shown). The expression of Fas was determined using a Coulter Epics flow cytometer.

Fas expression on theronts at different times post-harvest

To determine the effect of time of post-theront harvest on Fas expression, approximately 4000 theronts in 200 μL solution were added to each of 16 flow cytometer tubes and Fas expression assayed at 0, 0.5, 1 and 2 h after theront harvest. These tubes were divided into two subgroups. Theronts in one subgroup of tubes were incubated with PBS, serving as a negative control. In the other subgroup, theronts were incubated with FITC-Mab-Fas as described above. The expression of Fas was determined for 2000 theronts in each tube and two tubes for each time post-harvest. The assays were repeated twice and a total of 8000 theronts were evaluated for Fas expression at each time period.

Effect of theront concentration on Fas expression

Theronts were enumerated in five 10-μL samples of the theront solution using a Sedgewick-Rafter cell. After adjusting to 48 000 theronts mL^{-1} with filter-sterile tank water, eight 15-mL centrifuge tubes were filled with 0.5, 1, 2 or 4 mL of theront solution; two tubes were used for each concentration. Filter-sterilized tank water was added to each tube to bring the volume to 4 mL, so that theront concentrations were 6000, 12 000, 24 000 and 48 000 theronts mL^{-1}, respectively. After incubation at room temperature (22 ± 1 °C) for 1 h, theronts in each tube were concentrated to 200 μL at 60 ***g*** for 5 min. These tubes were divided into two subgroups, four tubes per subgroup. Theronts in one subgroup of tubes were incubated with FITC-Mab-Fas. In the other subgroup, theronts were incubated with PBS, serving as a negative control. The expression of Fas was determined for 2000 theronts in each tube and assays were repeated three times.

Blocking of Fas receptor on theronts by anti-Fas monoclonal antibodies

Two anti-Fas Mabs, mouse IgM from clones 7C11and CH11, purchased from Immunotect were used to further demonstrate the presence of Fas receptor on theronts. Five flow cytometer tubes were filled with 200 μL theront solution containing approximately 4000 theronts per tube. Theronts in these tubes were treated with 7C11 at 4 and 8 μg mL^{-1}, CH11 at 5 and 10 μg mL^{-1}, and PBS (pH 7.4) at 10 μL mL^{-1} at room temperature for 30 min, respectively. Then, 5 μL FITC-Mab-Fas was added to each tube and incubated for 15 min. The expression of Fas was determined for 2000 theronts in each tube and assays were repeated three times.

Effect of cutaneous antibody and Fas ligand on theront apoptosis

Fas ligand (FasL) used in this study was purchased from Sigma. Twenty 15-mL centrifuge tubes were filled with 3 mL theront solution containing approximately 1500 theronts mL^{-1} per tube. Then, theronts in each tube were treated by adding one of the following: FasL to 2 $\mu g\ mL^{-1}$, FasL to 5 $\mu g\ mL^{-1}$, immune skin culture fluid at a dilution of 1:10, non-immune skin culture fluid at a dilution of 1:10, and 1% bovine serum albumin (BSA in PBS, pH = 7.2) at a dilution of 1:10. Theront apoptosis was then determined after incubation at room temperature for 3 and 6 h. Theronts were washed with PBS and centrifuged at 60 ***g*** and 20 °C for 5 min. After discarding the supernatant (kept 200 μL) and adding 100 μL binding buffer, the theront suspension was transferred to a flow cytometer tube. The apoptosis in 2000 theronts was determined for each tube with the Annexin V Apoptosis Detection Kit and for two tubes for each treatment at each sampling time. The assays were repeated three times. The viable, apoptotic and necrotic theronts were expressed as percentages as in a previous study (Xu *et al.* 2005).

Correlation between Fas expression and theront apoptosis induced by immune skin culture fluid

After theronts were harvested as described above, 5 mL theront solution (22 000 theronts mL^{-1}) was placed into a 15-mL centrifuge tube. Five hundred microlitres of skin culture fluid from fish immune to Ich was added to the tube to make a 1:10 dilution. To determine the correlation between theront apoptosis and Fas expression, approximately 4000 theronts in 200 μL theront solutions were distributed to each of 16 flow cytometer tubes. Theront apoptosis and Fas expression were assayed simultaneously at 0, 0.5, 1 and 2 h after theronts were exposed to immune skin culture fluid. Sixteen tubes were divided into two 8-tube subgroups. Theronts in one subgroup of tubes were incubated with FITC-Mab-Fas and Fas expression was determined for 2000 theronts in each tube and for two tubes at each time. Theronts in the other eight tubes were used to determine theront apoptosis with the Annexin V Apoptosis Detection Kit. The assays were repeated twice and a total of 8000 theronts were evaluated for apoptosis and Fas expression at each time period.

Statistics

Duncan's multiple range test (SAS Institute 1989) was used to compare the percentage of Fas-positive theronts after Fas blocking by anti-Fas Mab and to analyse the effects of Fas ligand and skin culture fluid from fish immune to Ich on theront apoptosis. The correlation between theront apoptosis and Fas expression was evaluated using Pearson's correlation. Probabilities of 0.05 or less were considered statistically significant.

Results

Immune cutaneous antibody from skin culture fluid

The skin culture fluid from fish immune to Ich contained anti-Ich antibody at an immobilization titre of 1:64. No immobilization was observed for theronts exposed to the skin culture fluid from non-immune control fish. The ELISA titres were 1:128 for skin culture fluids from fish immune to Ich compared with 1:1 for non-immune fish.

Fas expression on theronts at different times post-harvest

Fluorescein isothiocyanate Mab-Fas reacted specifically with the Fas receptor on the theront surface and emitted green fluorescence. Fas-positive theronts were detected with a flow cytometer and more theronts expressed Fas as the time increased after harvest (Fig. 1). The percentages of Fas-positive theronts were 45.6%, 63.0%, 87.8% and 87.1%, respectively, when assayed at 0, 0.5, 1 and 2 h after harvest. Fas receptor was not detected for control theronts incubated with PBS.

Effect of theront concentration on Fas expression

After harvest and incubation at 24 °C for 1 h, only 19.6% of theronts expressed Fas on the cell surface in a concentration of 6000 theronts mL^{-1}. Fas-positive theronts increased significantly with increasing theront concentration ($P < 0.05$). The percentages of Fas-positive theronts were 38.1%, 47.7%, and 61.3% for theronts at concentrations of 12 000, 24 000 and 48 000 theronts mL^{-1}, respectively (Fig. 2).

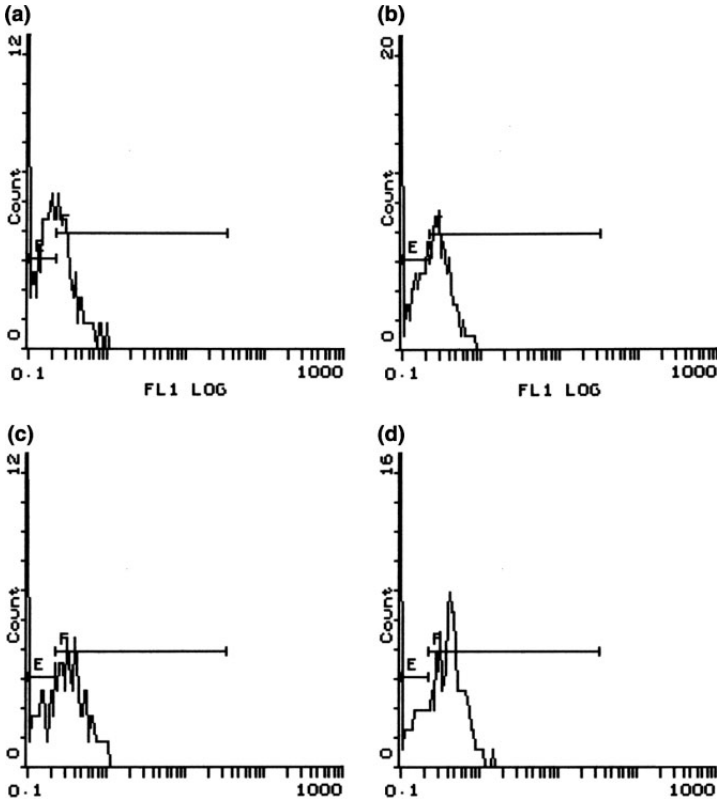

Figure 1 Fas expression on theronts was determined using fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against Fas receptor and by a Coulter Epics flow cytometer equipped with a 15 mW argon ion laser operating at 488 nm. The shift of theront peaks to the right of FL1 log from (a) to (d) shows the increase in Fas-positive theronts. Fas-positive theronts were 45.6%, 63.0%, 87.8% and 87.1%, respectively, when assayed at 0 h (a), 0.5 h (b), 1 h (c) and 2 h (d) post-harvest.

Blocking of Fas receptor on theronts by anti-Fas monoclonal antibodies

Both anti-Fas Mab 7C11 and CH11 bound to the Fas receptor on theronts and reduced the binding sites for FITC-Mab-Fas. The percentages of Fas-positive theronts were significantly lower in theronts treated with 7C11 or CH11 than in those treated with PBS (Table 1). Fas-positive theronts were also significantly less in treatments with higher concentrations of 7C11 or CH11 compared with lower concentrations ($P < 0.05$).

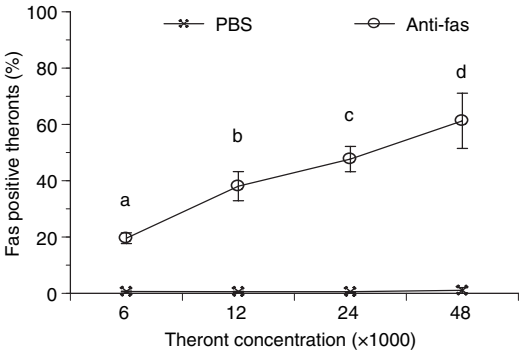

Figure 2 Fas-positive theronts significantly increased with increasing theront concentration. Each value is the mean percentage of Fas-positive theronts from three assays and vertical bars represent SD. Mean percentages with a different letter are significantly different ($P < 0.05$).

Effect of anti-Ich antibody or FasL on theront apoptosis

Viable theronts revealed low annexin V-FITC and low PI in flow cytometric analysis as they retained integrity of the cell membrane and asymmetry of their membrane phospholipids. Apoptotic theronts maintained the integrity of the cell membrane, but

Table 1 Percentage of Fas-positive theronts (mean ± SD) after blocking of Fas receptor on theronts by mouse anti-Fas monoclonal antibodies

Treatment	Concentration (μg mL^{-1})	Fas-positive theronts (%)
7C11	4	43.7 ± 10.1 a
7C11	8	32.3 ± 1.9 b
CH11	5	35.0 ± 6.4 b
CH11	10	21.5 ± 9.3 c
PBS	10	66.7 ± 12.1 d

Means followed by a different lower-case letter are significantly different ($P < 0.05$). PBS, phosphate-buffered saline.

Treatment	Concentration	Time (h)	Apoptosis (%)	Necrosis (%)	Viable (%)
Fas ligand	2 μg mL^{-1}	3	0.8 ± 0.2 a	2.8 ± 1.7 a	96.3 ± 5.1 a
Fas ligand	5 μg mL^{-1}	3	2.5 ± 1.9 a	8.0 ± 11.3 a	89.5 ± 13.2 b
Immune fluid	1:10	3	31.2 ± 11.1 b	31.3 ± 8.7 b	37.5 ± 11.3 c
Control fluid	1:10	3	8.2 ± 2.6 a	13.6 ± 12.2 c	78.2 ± 13.9 b
1% BSA	1:10	3	7.2 ± 5.8 a	8.4 ± 6.0 a	84.4 ± 11.9 b
Fas ligand	2 μg mL^{-1}	6	1.9 ± 1.3 a	7.5 ± 4.4 a	91.6 ± 4.6 b
Fas ligand	5 μg mL^{-1}	6	1.3 ± 0.5 a	12.2 ± 3.1 c	86.4 ± 13.5 b
Immune fluid	1:10	6	30.9 ± 10.2 b	43.5 ± 2.4 d	25.6 ± 8.7 c
Control fluid	1:10	6	10.3 ± 3.3 a	24.7 ± 4.9 c	65.1 ± 6.9 d
1% BSA	1:10	6	9.3 ± 8.2 a	17.4 ± 8.2 c	73.2 ± 6.3 b

Table 2 Effects of Fas ligand and skin culture fluid from fish immune to Ich on theront apoptosis at different times post-treatment

Viable, apoptotic and necrotic theronts are expressed as percentage (mean ± SD). Viable theronts revealed low annexin V-FITC (FITC) and low propidium iodide (PI) in flow cytometric analysis. Apoptotic theronts displayed high FITC and low PI and necrotic cells showed high FITC and high PI. Within a column, means are compared for 3 and 6 h post-treatment, respectively, and values followed by the same lower-case letter are not significantly different ($P > 0.05$). BSA, bovine serum albumin.

lost the asymmetry of their membrane phospholipids and consequently displayed high FITC and low PI. Necrotic theronts lost both integrity of the cell membrane and asymmetry of the membrane phospholipids and showed high FITC and PI. The effects of cutaneous antibodies in skin culture fluid from fish immune to Ich or FasL on theront apoptosis are presented in Table 2. The culture fluid from fish immune to Ich induced significantly higher apoptosis and necrosis in theronts compared with other treatments ($P < 0.05$). FasL did not induce apoptosis of theronts at concentrations of 2 and 5 μg mL^{-1}. No statistical difference was observed ($P > 0.05$) between theronts treated with FasL at concentrations of 2 or 5 μg mL^{-1}, culture fluid from non-immune fish at 1:10 or BSA as a control.

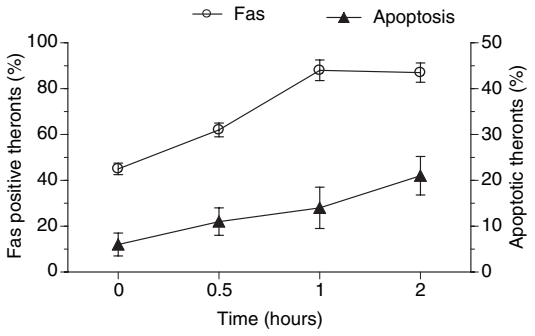

Figure 3 Correlation between Fas expression and theront apoptosis by exposure to skin culture fluid from channel catfish immune to Ich (correlation coefficient = 0.95, $P < 0.01$). The percentages of Fas-positive theronts (*y*-axis) were determined by immunofluorescence cytometry using the fluorescein isothiocyanate (FITC)-conjugated anti-Fas monoclonal antibody and apoptotic theronts (second *y*-axis) using FITC-conjugated annexin V. The assays for Fas receptor on theronts and theront apoptosis were conducted simultaneously and assays were repeated twice.

Correlation between Fas expression and theront apoptosis induced by immune skin culture fluid

Apoptotic theronts increased with time increment post-exposure to immune skin culture fluid. The mean percentages of apoptotic theronts were 6.2%, 10.7%, 16.7% and 21.5%, respectively, when assayed at 0, 0.5, 1 and 2 h. A highly significant ($P < 0.01$) positive correlation ($r = 0.95$) was noted between theront Fas expression and theront apoptosis induced by immune skin culture fluid (Fig. 3).

Discussion

Theronts showed higher apoptosis when exposed to the cutaneous antibody in skin culture fluid from fish immune to Ich than when exposed to skin culture fluid from non-immune fish. The results of this study further confirmed our previous results (Xu *et al.* 2005), where we noted that theront apoptosis occurred following exposure to the skin culture fluid from fish immune to Ich, which contained cutaneous antibody against the parasite. The apoptotic theronts showed characteristic chromatin condensation, nuclear fragmentation and PS externalization. There are two possible mechanisms for skin culture fluid to induce apoptosis of theronts. Cutaneous antibody in immune skin culture fluid may react with Fas receptor on theronts and activate their apoptotic pathway. We have noted that specific binding of the anti-Fas antibody to Fas receptors of theronts could trigger

theront apoptosis (D-H Xu, PH Klesius & CA Shoemaker, unpublished data). Further study is needed to determine whether cutaneous antibody in immune skin culture fluid binds to Fas receptor on theronts. The second possible mechanism is related to the immobilization of theronts. Cutaneous antibody in skin culture fluid from fish immune to Ich immobilizes and weakens theronts and further aggregates theronts into dense clumps (Xu *et al.* 2002). The high density of theronts enhances Fas expression on theronts, which in turn increases apoptosis of theronts.

The Fas receptor was detected on the theront surface using FITC-Mab-Fas and flow cytometry. Both time post-theront harvest and theront concentration influenced Fas expression of theronts. In this study, Fas-positive theronts significantly increased with time during *in vitro* incubation and with increasing theront concentration. The blocking of Fas receptor by anti-Fas Mabs 7C11and CH11 reduced the binding sites for FITC-Mab-Fas and resulted in a low number of Fas-positive theronts when determining Fas using flow cytometry. Fas has been reported on a variety of cell types in multicellular organisms, including thymocytes, activated T and B cells and some epithelial cells (Debatin 1998; Baran, Weglarczyk, Mysiak, Guzik, Ernst, Flad & Pryjma 2001).

Research into the role of Fas/FasL interaction in apoptosis has been conducted extensively for multicellular organisms (Nagata & Goldstein 1995). As one potential death activator, FasL binds to Fas receptors on the cell surface and signals the cell to start apoptosis. Triggering this pathway requires the cross-linking of Fas receptor on the cell surface with cell-expressing FasL or purified FasL (Baran *et al.* 2001). FasL-induced apoptosis has been established as a main pathway of apoptosis in multicellular organisms (Suda, Hashimoto, Tanaka, Ochi & Nagata 1997; Sawa, Nishimura, Ohyama, Takahashi, Takashiba & Murayama 1999), but it is not clear whether unicellular organisms have a similar pathway of apoptosis. In this study, we could not induce theront apoptosis with exogenous Fas ligand. Further study is needed to determine whether a different Fas ligand is involved (i.e. Fas ligand specific for parasitic infections) or if theront apoptosis is not Fas ligand mediated.

Apoptosis in multicellular organisms has received considerable attention because of the many roles it plays, such as in embryonic development, metamorphosis, elimination of cells that represent a threat to the organism, and regulation of immune function (Gao, Herndon, Zhang, Griffith & Ferguson 1998). Unlike their multicellular counterparts, unicellular organisms undergo apoptosis for the benefit of the entire population instead of an individual organism (Deponte & Becker 2004). Apoptosis in unicellular organisms plays a major role in controlling populations by sacrificing undesirable cells (Tan *et al.* 2001; Al-Olayan *et al.* 2002). Lee *et al.* (2002) noted that differentiation, deprivation of nutrients essential to growth or increased cell density, could induce apoptosis of the unicellular parasite *Leishmania* by self-destruction of some cells for the benefit of parasite survival. Other factors have also been reported to induce the apoptosis of unicellular organisms, including poor environmental conditions (Vardi *et al.* 1999), intercellular signalling (Murphy & Welburn 1997; Christensen, Chemnitz, Straarup, Kristiansen, Wheatley & Rasmussen 1998), drugs and antibody against the organism (Nasirudeen, Singh, Yap & Tan 2001). In this study, both numbers of Fas-positive theronts and apoptotic theronts increased with time increment post-exposure to skin culture fluid from fish immune to Ich. Some theronts may die because of apoptosis to protect other members in the same population under adverse conditions.

In summary, Fas receptor was detected on the surface of Ich theronts, and Fas-positive theronts increased significantly with time of *in vitro* incubation and with increasing theront concentration. Cutaneous anti-Ich antibody in skin culture fluid from fish immune to Ich induced theront apoptosis but Fas ligand did not. Apoptosis of theronts induced by immune cutaneous antibody appears to be correlated with the expression of Fas on the surface of Ich theronts.

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